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Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery

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Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein-9 (CRISPR-Cas9) can be used as an efficient tool for genome editing in potato (*Solanum tuberosum*). From both a scientific and a regulatory perspective, it is beneficial if integration of DNA in the potato genome is avoided. We have implemented a DNA-free genome editing method, using delivery of CRISPR-Cas9 ribonucleoproteins (RNPs) to potato protoplasts, by targeting the gene encoding a granule bound starch synthase (GBSS, EC 2.4.1.242). The RNP method was directly implemented using previously developed protoplast isolation, transfection and regeneration protocols without further adjustments. Cas9 protein was preassembled with RNA produced either synthetically or by in vitro transcription. RNP with synthetically produced RNA (cr-RNP) induced mutations, i.e. indels, at a frequency of up to 9%, with all mutated lines being transgene-free. A mutagenesis frequency of 25% of all regenerated shoots was found when using RNP with in vitro transcriptionally produced RNA (IVT–RNP). However, more than 80% of the shoots with confirmed mutations had unintended inserts in the cut site, which was in the same range as when using DNA delivery. The inserts originated both from DNA template remnants from the in vitro transcription, and from chromosomal potato DNA. In 2–3% of the regenerated shoots from the RNP-experiments, mutations were induced in all four alleles resulting in a complete knockout of the GBSS enzyme function.

Introduction

Precision genome editing, through clustered regularly interspaced short palindromic repeats and CRISPR-associated protein-9 (CRISPR-Cas9), is performed by directing a guide RNA complementary to a defined target region, together with a CRISPR-associated endonuclease, Cas9, for double-stranded DNA cleavage (Jinek et al. 2012). For plant research and breeding, CRISPR-Cas9 is still a very recent method: optimization to increase efficiency and specificity of the method is receiving a lot of attention from different research groups in the field. Double-strand DNA breaks can result in unintended DNA insertions from plasmid DNA or from the plant genome occurring at a high frequency

Abbreviations – CRISPR-Cas9, clustered regularly interspaced short palindromic repeats and CRISPR-associated protein-9; GBSS, granule bound starch synthase; HRFA, high-resolution fragment analysis; Indels, inserts and/or deletions; NHEJ, non-homologous end joining; PAM, protospacer adjacent motif; RNP, ribonucleoprotein; TALEN, transcription activator-like effector nuclease; WGS, whole genome sequencing.

(Gorbunova and Levy 1997). Avoidance of unintended DNA insertions is one important objective for the optimization of the method, especially if the CRISPR-Cas9 tools are used in commercial crop breeding. Genome edited plants, with mutations in the form of small inserts or deletions (indels) are indistinguishable from plants containing naturally or conventionally induced mutations; it has therefore been argued that they do not differ from classically bred crops in terms of health and environmental issues (Hartung and Schiemann 2014). Studies have shown that delivering CRISPR-Cas9 as ribonucleoproteins (RNPs) into cells is a promising alternative to using DNA, with the added potential of generating transgene-free targeted genome edits efficiently (Cho et al. 2013, Kim et al. 2014, Woo et al. 2015, Liang et al. 2017). RNPs have been described as being more specific and to act more rapidly since the complex can act immediately without the need of intracellular transcription and translation; a clear reduction in off-target mutations has also been demonstrated (Cho et al. 2013, Svitashev et al. 2016, Liang et al. 2017).

Cultivated potato (*Solanum tuberosum*) is a tetraploid and largely heterozygous crop, and its tetrasomic inheritance makes potato research and breeding through traditional crossbreeding a challenge (Muthoni et al. 2015). By specific applications of genome editing, one or a few traits can be added to a commercial variety, hence crossbreeding can be avoided. In recent years, genome editing through either TALEN or CRISPR-Cas9 has been used to study and develop commercially important traits in potato; traits which would otherwise be difficult to include through traditional breeding technologies (Sawai et al. 2014, Clasen et al. 2016, Andersson et al. 2017). However, when using DNA transfection of protoplasts, unintended inserts have been detected that originated from the plasmid DNA used (Clasen et al. 2016, Andersson et al. 2017). In the present study, therefore, CRISPR-Cas9 was further developed as a potato breeding method by implementing RNP delivery in protoplasts to decrease or eliminate the presence of unintended inserts in progeny. The successful development of the well-studied trait amylopectin starch was used as a marker for the functionality of the RNP technology (Kuipers et al. 1991, Kuipers et al. 1994, Andersson et al. 2003). A native starch is a mixture of amylopectin and amylose. Amylopectin starch potatoes were developed by eliminating the amylose formation by knocking out the sole enzyme responsible for synthesis of amylose, a granule bound starch synthase (GBSS). The high frequency of GBSS knockout genotypes produced in this study shows the potential of CRISPR-Cas9 RNP technology as a future potato breeding method.

Material and methods

Protoplast isolation, transfection and regeneration

Protoplasts were isolated from *S. tuberosum* cultivar Kuras. In vitro propagation, protoplast isolation, transfection and regeneration were performed as described in Andersson et al. (2017). For each single experiment, 100 000 protoplasts were incubated with either 25% w/v PEG 4000 (Duchefa, Haarlem, The Netherlands) and DNA or RNP for 3 min, or with 40% w/v PEG and RNP for 30 min.

Exon 9 in the *GBSS* gene (Fig. 1A) was targeted for induced mutation at a previously defined region named GT4 (Andersson et al. 2017). Protoplasts were transfected with preassembled gRNA saturated RNP or with plasmid DNA. Two different RNP combinations were used, an Alt-R® CRISPR-Cas9 system (IDT, Coralville, IA) and a GeneArt™ system (Thermo Fisher Scientific, Waltham, MA). For the Alt-R® CRISPR-Cas9 system, GT4-crRNA was synthetically produced and preassembled with Alt-R® CRISPR-Cas9 tracrRNA and Alt-R® S.p.Cas9 Nuclease 3NLS (cr-RNP). For the GeneArt™ system, GT4-RNA was produced through in vitro transcription and DNaseI treatment following GeneJET™ RNA purification using a Precision gRNA Synthesis Kit according to the suppliers' instruction and preassembled with GeneArt™ Platinum™ Cas9 Nuclease (IVT–RNP). The RNA concentration from the in vitro transcription, as well as the RNA:DNA ratio, were quantified using Trinean DropSense™ 16 (Tectum, Nacka, Sweden). For the cr-RNP experiments, 2.0 nmol of GT4-crRNA and 2.0 nmol of tracrRNA together with 0.03 nmol (5 µg) of Cas9 protein was transfected to the protoplasts. For the IVT–RNP experiments, 0.13 nmol of the in vitro transcribed GT4-RNA together with 0.03 nmol (5 µg) of Cas9 Nuclease was used for transfection. As a comparator, 5 µg plasmid DNA, harbouring both the Cas9 and sgRNA (pE-GT4) described previously (Andersson et al. 2017), was used for transfection. Care was taken to only recover one shoot from each callus to avoid studying clones.

Mutation identification and characterisation

Leaf tissue from regenerated shoots was analysed using high-resolution fragment analysis (HRFA) as previously described (Andersson et al. 2017). A PCR fragment of 145 bp spanning the GT4 target site was amplified using primers StGBSS(GT4)f-FAM and StGBSS(GT4)r (Andersson et al. 2017) and analysed on a 3500 Genetic analyser (Thermo Fisher Scientific, Waltham, MA). Indels were detected to a resolution of 1 bp, and inserts up to approximately 500 bp were detected in the same analysis. As a size marker, GeneScan 600LIZ was

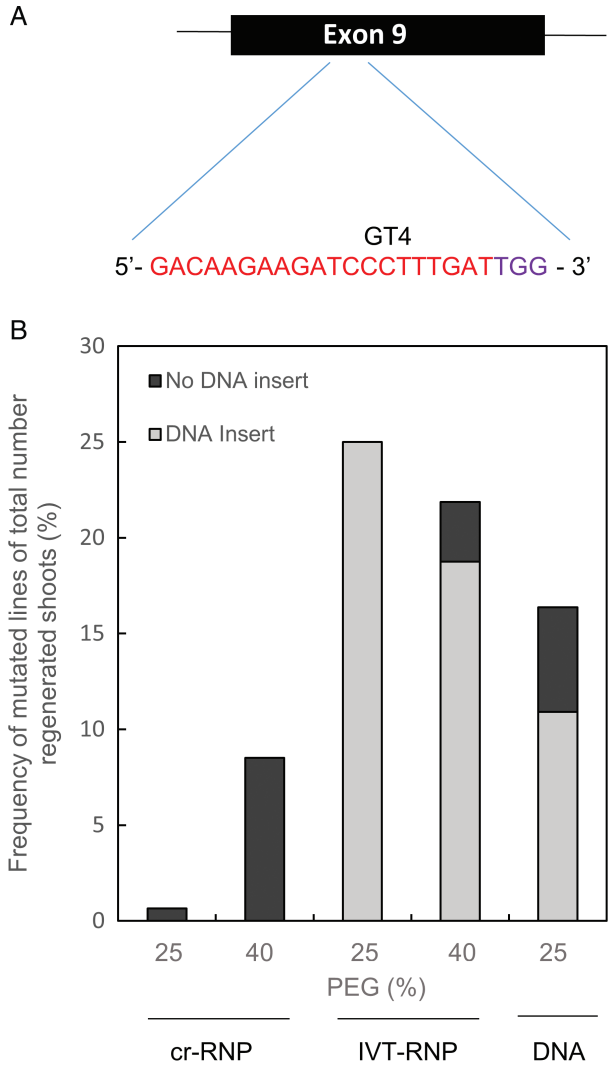

Fig. 1. Targeted mutagenesis in potato using RNP CRISPR-Cas9. (A) Target region GT4 in *GBSS* exon 9 shown in red, PAM sequence shown in purple. (B) Frequency (%) of lines with at least one mutated allele, on the total number of regenerated shoots from each experiment, detected using HRFA. RNP including synthetically produced RNA (cr-RNA), RNP including in vitro transcribed RNA (IVT–RNP) and plasmid (DNA) transfected to potato protoplast using 25 or 40% PEG. $n = 4–420$.

used (Thermo Fisher Scientific). Primers StGBSS(GT4)f and StGBSS(GT4)r were used for amplifying the same region spanning the GT4 target followed by cloning and Sanger sequencing (GATC Biotech, Konstanz, Germany) as described previously (Andersson et al. 2017). Inserts of Cas9 coding region in shoots regenerated from the pE-GT4 DNA experiment were detected by multiplexing the HRFA analysis with PCR amplification of a 454 bp fragment using primers Cas9_2f-Hex: GCTGGAGAGCTTCAGAAGGGA and Cas9_2r: CTCCTCCAAGCTGAGAAAGATCG, where the 5′ end of the forward primer was labelled with the fluorescent dye Hex (Sigma-Aldrich, St. Louis, MO). The frequencies were calculated based on all regenerated shoots from each experimental setup.

Microtuber propagation and starch quality determination

Potato lines with mutations in all four alleles were selected and cultivated to produce microtubers. (Andersson et al. 2003). Starch from developed microtubers was analysed and high amylopectin quality confirmed by iodine staining and light microscopy (Leica microsystem DM LB, Wetzlar, Germany).

Results

Induced mutations through RNP delivery in potato protoplasts

A region in exon 9 of the gene encoding GBSS, named GT4 (Fig. 1A), was targeted to induce mutations using delivery of CRISPR-Cas9 components in the form of pre-assembled RNP to isolated potato protoplasts. The potential of inducing mutations in potato through RNP was studied using both 25 and 40% PEG-mediated protoplast transfection. Plasmid DNA encoding the CRISPR-Cas9 editing components, as a comparator, was delivered using 25% PEG-mediated protoplast transfection. Cas9 protein was pre-assembled with either synthetically produced crRNA and tracrRNA, cr-RNP, or in vitro transcribed RNA, IVT–RNP. Mutations were detected using HRFA-analysis (Fig. 2A) and the mutagenesis frequency was calculated based on the total number of regenerated shoots from callus formed by transfected protoplasts (Figs 1B and 2B). Mutated shoots were further studied using PCR, cloning and sequencing to confirm results obtained from HRFA analysis (Fig. 2C). The frequency of lines with at least one mutated allele, was 9% based on 33 regenerated shoots in the cr-RNP study using the 40% PEG experimental conditions, while only 1% of 420 regenerated shoots was found to be mutated when using 25% PEG. Even though PEG can be toxic to cells in high concentrations, the divergently high regeneration seen in the latter experiment is more likely explained by an incomplete transfection and hence the reason for the low frequency of mutations found. For the cr-RNP experiments, no inserts were detected in any of the regenerated shoots studied. With IVT–RNP, the frequencies of shoots with at least one allele mutated were similar, independent of the experimental conditions used, resulting in 25 and 22% of 4 and 32 regenerated shoots, respectively (Fig. 1B). The mutagenesis frequency using DNA delivery was

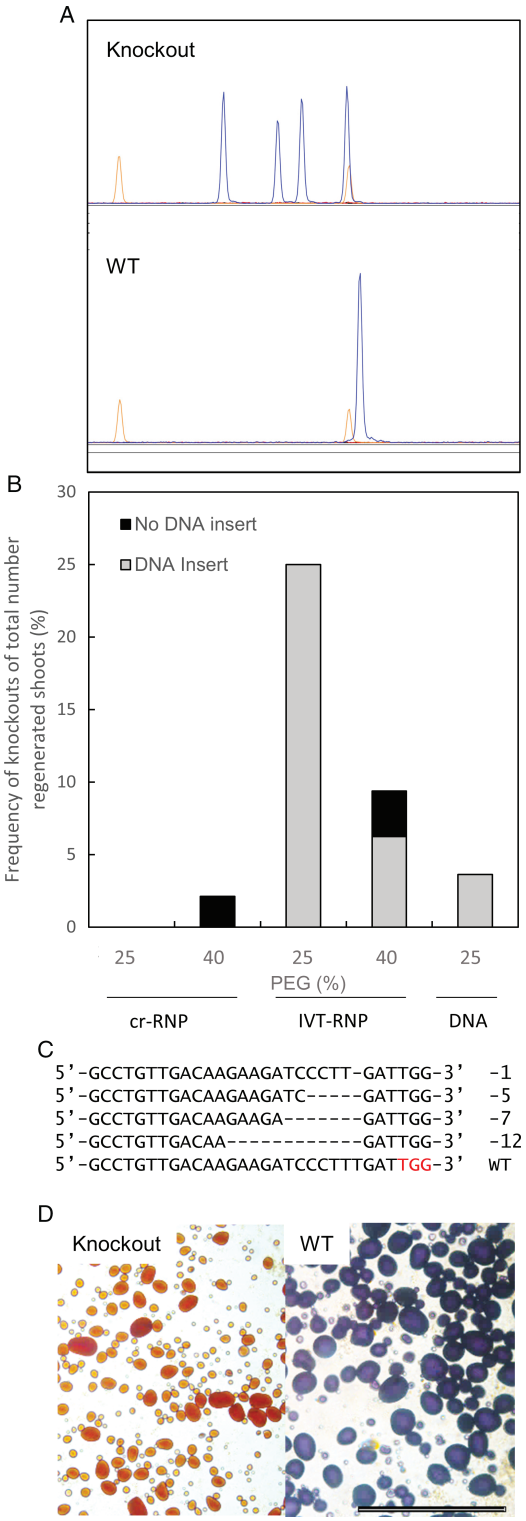

Fig. 2. Legend on next column.

16% of 55 regenerated shoots using the 25% PEG experimental conditions (Fig. 1B). The mutated lines (86–100%) from the IVT–RNP experiment were found to have inserts in the cut site based on HRFA analysis, while when using DNA delivery, inserts were confirmed based on Cas9 transgene integration to a frequency of 67% of all mutated lines (Fig. 1B). The origin of inserts was analysed by PCR amplification covering the GT4 cut site, followed by sequencing. From the IVT–RNP studies, inserts of both the gRNA DNA template, as well as chromosomal DNA from random parts of the genome, were detected in the studied lines; while in the DNA delivery study, plasmid DNA was the origin of the inserts. Despite DNAseI treatment and RNA purification, the in vitro transcribed RNA was found to contain DNA contamination. The amount of DNA in the sample was somewhat difficult to quantify, and the DNA:RNA ratio varied from 1:7 (in 390 ng/µl RNA) to 1:37 (in 28 ng/µl RNA) depending on dilution factor.

Development of transgene-free knockouts

Two lines with mutations in all four alleles and with no unintended DNA inserts detected were developed, one from the cr-RNP experiment and one from the IVT–RNP experiment. These yielded a knockout frequency of 2% for the cr-RNP and 3% for the IVT–RNP experiments, based on total number of regenerated shoots when using the 40% PEG experimental condition (Fig. 2B). Lines with mutations in all four alleles were also detected when using IVT–RNP and DNA with 25% PEG experimental conditions to a frequency of 25 and 3%, with all lines confirmed as containing unintended DNA inserts (Fig. 2B). Lines with mutations in all four alleles, confirmed by HRFA analysis (Fig. 2A) as well as Sanger sequencing (Fig. 2C), were cultivated in vitro to produce microtubers. Starch granules from the microtubers were subjected to iodine-based staining and light microscopy to analyse starch quality. Absence of amylose was evident from the red–brown stained starch from the knockout lines while starch from the wild type was stained blue (Fig. 2D).

Fig. 2. Development of knockout lines. (A) HRFA analysis of knockout line (top) and wild type (WT, bottom). (B) Frequency (%) of four-allele mutated lines, on the total number of regenerated shoots from each experiment, detected using HRFA and Sanger sequencing. RNP including synthetically produced RNA (cr-RNA), RNP including in vitro transcribed RNA (IVT–RNP) and plasmid (DNA) transfected to potato protoplast using 25 or 40% PEG. $n = 4–420$. (C) Genotyping of all four alleles in a knockout line. PAM sequence shown in red in WT sequence (bottom). (D) Phenotyping of starch by iodine staining and light microscopy of knockout line yielding amylopectin starch quality (left) and WT (right). Scale bar 100 µm.

Discussion

Whether or not genome edited plants should fall under the legislation of genetically modified organisms (GMO) as regulated plant events has been a point of discussion in the EU for many years (Hartung and Schiemann 2014, Jones 2015). The lack of a clear decision from the European Union on this issue has resulted in some member states making a temporary decision based on their own interpretation of the existing GMO legislation. For example, in 2015, the Swedish Board of Agriculture gave clearance for Swedish researchers to grow genome edited transgene-free *Arabidopsis thaliana* plants in a field trial without the need of a specific GMO-permit (Eriksson et al. 2017). Transgene-free genome edited plants can be produced using CRISPR-Cas9 or similar techniques by either developing a transgenic plant followed by outcrossing of the integrated DNA, or by using a transient system as for example protoplast transfection or microprojectile bombardment. For potato, a system yielding no foreign DNA integration would be the preferred method, in order to avoid crossbreeding of this highly heterozygous tetraploid crop, which could then result in inbreeding depression (Xu et al. 2011).

In a previous study by Andersson et al. (2017), CRISPR-Cas9 was implemented as a potato breeding method in which the delivery of the mutagenizing components was in the form of plasmid DNA to protoplasts. That study resulted in a mutagenesis frequency of up to 12%, with 10% of the lines having unintended plasmid DNA integrated in the cut site of target regions. It should be noted that the analysis method used in that study could only detect inserts of up to approximately 500 bp, hence the presence of larger inserts of the 9000 bp vector was not investigated. In this study, the mutagenesis frequency was 16% when using DNA delivery (Fig. 1B). However, two thirds of the mutated lines were found to have unintended insertions confirmed by a Cas9 fragment integration, and the figure could be even higher since the full vector was not covered in the analysis. This high percentage of inserts are in line with what has previously been published for double strand breaks in potato, by using TALEN and DNA transfection of protoplasts, where approximately 60% of the regenerated lines having DNA integration was presented (Clasen et al. 2016). A similarly high frequency of DNA integration has also been observed in other crops: for example, in wheat, where mutants were developed by bombardment of immature embryos and seedlings developed in the absence of selection, resulting in a 40–60% frequency of DNA inserts (Zhang et al. 2016).

To eliminate the possibility of unintended DNA inserts, the use of pre-assembled CRISPR-Cas9 RNP for protoplast transfection was implemented in this study, by targeting the *GBSS* gene (Fig. 1A). Two types of RNPs were studied: cr-RNP, where the guide RNA was synthetically produced and IVT–RNP, where the guide RNA was produced through in vitro transcription. The mutagenesis frequency when using cr-RNP under 40% PEG-mediated transfection conditions was as high as 9%, with all regenerated lines being transgene-free (Fig. 1B). This frequency is comparable to what has been reported using RNP in maize, with a mutagenesis frequency of 2–10% (Svitashev et al. 2016), and wheat, yielding a mutagenesis frequency of 2–4% (Liang et al. 2017) based on regenerated shoots.

By using IVT, the insert integration frequency was similar to that when using DNA (Fig. 1B). Studying the origin of the inserts revealed that the majority came from remnants of DNA template for the in vitro transcription. Furthermore, the majority of the DNA was inserted without additional indels of the endogenous DNA connected to the cut site. Thus, it could be assumed that the insertions contributed to the higher frequency of mutagenesis observed for IVT–RNP. Quantifying DNA contamination in the RNA sample was difficult, most probably due to interference from the high RNA concentration during measurements. In an effort to make the IVT–RNP completely DNA-free, we varied the amount of DNA template as well as the amount and time available for DNAse treatment. Even though the DNA:RNA ratio was improved, it was in our hands still not possible to produce clean RNA samples (results not shown). In addition to the template DNA inserts, a few inserts were found to be genomic DNA of random parts of the potato genome. The detection limit of the HRFA analysis excludes large size inserts, while covering DNA template inserts and genomic rearrangements of up to 500 bp. The high frequency of DNA inserts in this IVT–RNP study is noteworthy, since the DNA contamination is probably rather low. However, the origin of the inserts is expectedly from double strand break repairs, since plasmid DNA and insertions of chromosomal DNA were detected in early studies (Gorbunova and Levy 1997). In the same study, DNA was also found inserted as reshuffled multiple regions of the plasmid. In the study by Andersson et al. (2017), sequence analysis of small fragments inserted at Cas9 cut sites were found to be random parts of plasmid DNA greatly varying in size. The unpredicted insert structure that could arise from large plasmids can make detection problematic when applying commonly used techniques such as PCR or Southern blotting in covering the full possibilities of inserts. Whole genome sequencings (WGS) is probably

the most efficient way to ensure that no integration of foreign DNA has occurred; however, this requires computational work and access to good bioinformatics tools.

The lower mutagenesis frequency found using cr-RNP, compared to the experiments yielding unintended inserts (both by using IVT–RNP and DNA) could indicate a high percentage of precise end joining after Cas9 cutting. This notion is further strengthened by the observation that the unintended DNA inserts usually had no additional indels associated with the cut site. A strong preference for precise ligation in cells repaired by non-homologous end joining (NHEJ) after dual Cas9 cuttings has previously been described, and figures of up to approximately 70% perfect ligations have been presented (Nelson et al. 2016).

From both the cr-RNP and the IVT–RNP experiments, knockout plants without any DNA integration were found (Fig. 2B). Hence, lines of potential commercial importance (here defined as having an introduced trait without DNA integration) were obtained from both RNP experiments. The amylopectin trait was confirmed in the knockouts by staining starch from microtubers with Iodine (Fig. 2D). Amylopectin starch granules stains red–brown, while starch granules containing only low levels of GBSS activity and small amounts of amylose stains blue (Visser et al. 1991). The knockout success rate of 2–3% in a tetraploid crop would probably be adequate for a commercial breeding programme. However, it should be remembered that somaclonal variation sometimes does occur when shoots are regenerated from protoplasts (Shepard et al. 1980, Larkin and Scowcroft 1981) and several knockout lines will therefore be needed to select a line of commercial quality. Further development of the method with the aim of yielding an even higher ratio of knockouts would therefore be welcome. The RNP complexes were here preassembled using a high gRNA:Cas9 excess molar ratio, which could be further optimised in future studies.

To summarise, in the present study, we have shown that RNP-delivery of CRISPR-Cas9 tools as a potato breeding method has good potential in yielding commercial lines without unintended DNA integration. At the same time, the importance of using a pure RNA sample in the RNP complex became obvious. Here, the RNP method could be directly implemented using a previously developed protoplast isolation, transfection and regeneration method, without any further modifications. We therefore propose RNP to be used instead of DNA, since it yields a high frequency of transgene-free mutated lines, so significantly simplifying analysis and selection of lines.

Author contributions

M. A. and P. H. conceived and designed research. M. A., A.-S. F., P. O., H. T., N. O., and M. G. designed and conducted experiments. M. S. supervised the research. M. A. and P. H. wrote the manuscript. All authors read and approved the manuscript.

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References

Andersson M, Trifonova A, Andersson AB, Johansson M, Bulow L, Hofvander P (2003) A novel selection system for potato transformation using a mutated AHAS gene. Plant Cell Rep 22: 261–267

Andersson M, Turesson H, Nicolia A, Falt AS, Samuelsson M, Hofvander P (2017) Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. Plant Cell Rep 36: 117–128

Cho SW, Lee J, Carroll D, Kim JS, Lee J (2013) Heritable gene knockout in *Caenorhabditis elegans* by direct injection of Cas9-sgRNA ribonucleoproteins. Genetics 195: 1177–1180

Clasen BM, Stoddard TJ, Luo S, Demorest ZL, Li J, Cedrone F, Tibebu R, Davison S, Ray EE, Daulhac A, Coffman A, Yabandith A, Retterath A, Haun W, Baltes NJ, Mathis L, Voytas DF, Zhang F (2016) Improving cold storage and processing traits in potato through targeted gene knockout. Plant Biotechnol J 14: 169–176

Eriksson D, Brinch-Pedersen H, Chawade A, Holme IB, Hvoslef-Eide TAK, Ritala A, Teeri TH, Thorstensen T (2017) Scandinavian perspectives on plant gene technology: applications, policies and progress. Physiol Plant 162: 219–238

Gorbunova V, Levy AA (1997) Non-homologous DNA end joining in plant cells is associated with deletions and filler DNA insertions. Nucleic Acids Res 25: 4650–4657

Hartung F, Schiemann J (2014) Precise plant breeding using new genome editing techniques: opportunities, safety and regulation in the EU. Plant J 78: 742–752

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337: 816–821

Jones HD (2015) Regulatory uncertainty over genome editing. Nat Plants 1: 3

Kim S, Kim D, Cho SW, Kim J, Kim JS (2014) Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res 24: 1012–1019

Kuipers GJ, Vreem JTM, Meyer H, Jacobsen E, Feenstra WJ, Visser RGF (1991) Field-evaluation of antisense RNA mediated inhibition of GBSS gene-expression in potato. Euphytica 59: 83–91

Kuipers AGJ, Soppe WJJ, Jacobsen E, Visser RGF (1994) Field-evaluation of transgenic potato giants expressing an antisense granule-bound starch synthase gene - increase of the antisense effect during tuber growth. Plant Mol Biol 26: 1759–1773

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell-cultures for plant improvement. Theor Appl Genet 60: 197–214

Liang Z, Chen K, Li T, Zhang Y, Wang Y, Zhao Q, Liu J, Zhang H, Liu C, Ran Y, Gao C (2017) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat Com 8: 14261

Muthoni J, Kabira J, Shimelis H, Melis R (2015) Tetrasomic inheritance in cultivated potato and implications in conventional breeding. Austr J Crop Sci 9: 185–190

Nelson CE, Hakim CH, Ousterout DG, Thakore PI, Moreb EA, Rivera RMC, Madhavan S, Pan XF, Ran FA, Yan WX, Asokan A, Zhang F, Duan DS, Gersbach CA (2016) In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. Science 351: 403–407

Sawai S, Ohyama K, Yasumoto S, Seki H, Sakuma T, Yamamoto T, Takebayashi Y, Kojima M, Sakakibara H, Aoki T, Muranaka T, Saito K, Umemoto N (2014) Sterol side chain reductase 2 is a key enzyme in the biosynthesis of cholesterol, the common precursor of toxic steroidal glycoalkaloids in potato. Plant Cell 26: 3763–3774

Shepard JF, Bidney D, Shahin E (1980) Potato protoplasts in crop improvement. Science 208: 17–24

Svitashev S, Schwartz C, Lenderts B, Young JK, Mark Cigan A (2016) Genome editing in maize directed by CRISPR–Cas9 ribonucleoprotein complexes. Nat Com 7: 13274

Visser RGF, Somhorst I, Kuipers GJ, Ruys NJ, Feenstra WJ, Jacobsen E (1991) Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs. Mol Gen Genet 225: 289–296

Woo JW, Kim J, Il Kwon S, Corvalan C, Cho SW, Kim H, Kim SG, Kim ST, Choe S, Kim JS (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotech 33: 1162–U1156

Xu X, Pan SK, Cheng SF, Zhang B, Mu DS, Ni PX, Zhang GY, Yang S, Li RQ, Wang J, Orjeda G, Guzman F, Torres M, Lozano R, Ponce O, Martinez D, De la Cruz G, Chakrabarti SK, Patil VU, Skryabin KG, Kuznetsov BB, Ravin NV, Kolganova TV, Beletsky AV, Mardanov AV, Di Genova A, Bolser DM, Martin DMA, Li GC, Yang Y, Kuang HH, Hu Q, Xiong XY, Bishop GJ, Sagredo B, Mejia N, Zagorski W, Gromadka R, Gawor J, Szczesny P, Huang SW, Zhang ZH, Liang CB, He J, Li Y, He Y, Xu JF, Zhang YJ, Xie BY, Du YC, Qu DY, Bonierbale M, Ghislain M, Herrera MD, Giuliano G, Pietrella M, Perrotta G, Facella P, O'Brien K, Feingold SE, Barreiro LE, Massa GA, Diambra L, Whitty BR, Vaillancourt B, Lin HN, Massa A, Geoffroy M, Lundback S, DellaPenna D, Buell CR, Sharma SK, Marshall DF, Waugh R, Bryan GJ, Destefanis M, Nagy I, Milbourne D, Thomson SJ, Fiers M, Jacobs JME, Nielsen KL, Sonderkaer M, Iovene M, Torres GA, Jiang JM, Veilleux RE, Bachem CWB, de Boer J, Borm T, Kloosterman B, van Eck H, Datema E, Hekkert BTL, Goverse A, van Ham R, Visser RGF, Potato Genome Sequencing C (2011) Genome sequence and analysis of the tuber crop potato. Nature 475: 189-U194

Zhang Y, Liang Z, Zong Y, Wang YP, Liu JX, Chen KL, Qiu JL, Gao CX (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat Com 7: 12617

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